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A temperature sensitive p210 BCR-ABL mutant defines the primary consequences of BCR-ABL tyrosine kinase expression in growth factor dependent cells

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The Philadelphia translocation commonly observed in chronic myeloid leukaemia (CML) and a proportion of cases of acute leukaemia results in the creation of a chimeric fusion protein, BCR-ABL. The fusion protein exhibits an elevated tyrosine kinase activity as compared to normal ABL. Using a temperature sensitive mutant of p210 BCR-ABL (ts-p210) we find that the primary effect of BCR-ABL expression in an IL-3 dependent cell line is to prolong survival following growth factor withdrawal; only a small proportion of cells remain viable and rapidly evolve to complete growth factor independence. During passage in the presence of IL-3 at the temperature permissive for kinase activity, ts-p210 expressing cultures become dominated by completely growth factor independent cells within 10–30 days. There is also a significant difference between BCR-ABL and IL-3 mediated signalling with respect to the MAP kinase pathway; in contrast to IL-3 stimulation or v-ABL expression, BCR-ABL does not signal ERK 2 (MAP 2 kinase) activation, underlining the apparent inability of BCR-ABL to deliver an immediate proliferative signal in Ba/F3 cells. Our data suggest that growth factor independence does not simply reflect the convergence of BCR-ABL and IL-3 mediated signalling pathways and its development, at least in Ba/F3 cells, requires prolonged exposure to BCR-ABL kinase activity. We suggest that the myeloid expansion characteristic of CML may result from the prolongation of survival of myeloid progenitor cells under conditions of limiting growth factor rather than their uncontrolled proliferation.

Key words: ABL/BCR/growth factor dependence/MAP kinase/tyrosine kinase

Introduction

Alterations in the ABL tyrosine kinase proto-oncogene are critical in the pathogenesis of the human haematopoietic malignancies, chronic myeloid leukaemia (CML) and Philadelphia-positive acute lymphoblastic leukaemia (Ph-positive ALL) (for reviews see Clark *et al.*, 1989; Allen *et al.*, 1992). Initially identified as the normal cellular homologue of the viral v-ABL gene of the Abelson murine leukaemia virus (AbMLV) (Wang and Baltimore, 1983), ABL is activated in these diseases by the replacement of its first exon with sequences from the BCR gene. This results in a BCR-ABL fusion protein with enhanced tyrosine kinase activity (Konopka *et al.*, 1984) which has been shown to induce various haematopoietic malignancies in transgenic and bone marrow reconstituted mice (Daley *et al.*, 1990; Elefanty *et al.*, 1990; Heisterkamp *et al.*, 1990; Kelliher *et al.*, 1990). The BCR-ABL proteins associated with CML and Ph-positive ALL differ in the contribution of BCR sequences to their N-termini; the p190 BCR-ABL protein more commonly associated with Ph-positive ALL (Chan *et al.*, 1987) contains 426 exon 1 encoded BCR residues (Hermans *et al.*, 1987), while the p210 protein found in both CML and Ph-positive ALL contains either 902 or 927 BCR amino acid residues (Heisterkamp *et al.*, 1985; Hariharan and Adams, 1987).

CML is characterized by an initial chronic phase, resembling a myeloproliferative disorder in many of its features, with elevation of peripheral granulocyte counts. Although activated ABL oncogenes have been shown to abrogate the growth factor requirement of several myeloid and lymphoid cell lines (Daley and Baltimore, 1988; Laneuville and Sullivan, 1991; Sirard *et al.*, 1994), altered growth factor responsiveness of Ph-positive haemopoietic progenitors has not been demonstrated, and chronic phase CML cells retain growth factor requirement and normal proliferative responses to IL-3, GM-CSF and G-CSF (Moore *et al.*, 1973; Goldman *et al.*, 1974; Metcalf *et al.*, 1974; Lansdorp *et al.*, 1985). This suggests that the myeloid expansion characteristic of chronic phase CML may result from prolongation of cell survival rather than uncontrolled proliferation. The chronic phase inevitably progresses to a terminal blast crisis with accumulation of immature blast cells of lymphoid or myeloid lineage (Golde *et al.*, 1974); this stage of the disease resembles an acute leukaemia and is associated with the accumulation of secondary genetic abnormalities (Blick *et al.*, 1987; Hagemeijer, 1987). BCR-ABL expression is thought to have an initiating role in the pathogenesis of CML, and transformation to an acute leukaemia presumably arises as a consequence of additional genetic mutation(s) in a cell of the Ph-positive clone (Zalcberg *et al.*, 1986).

In an effort to define the primary consequences of BCR-ABL expression in growth factor dependent haematopoietic cells and to distinguish these from secondary events associated with growth factor independence/transformation, we constructed a temperature sensitive conditional mutant of p210 BCR-ABL (ts-p210) and introduced this gene into the IL-3 dependent Ba/F3 cell line. From our observations, we conclude that abrogation of growth factor dependence is not a primary effect of BCR-ABL expression; cells require prolonged exposure to BCR-

ABL tyrosine kinase activity for the development of growth factor independence, reflecting a possible requirement for additional co-operating genetic events in the abrogation of growth factor dependence by BCR−ABL.

Extracellular signal-regulated kinases (ERKs)/mitogen activated kinases (MAP kinases) are a family of protein serine/threonine kinases that have been shown to be critical components of tyrosine kinase mediated signal transduction pathways involved in the induction of proliferation and differentiation in a wide variety of cell types (Pelech and Sanghera, 1992). We examined the involvement of the MAP kinase pathway in BCR−ABL mediated signal transduction and found that, in contrast to IL-3 stimulation of Ba/F3 and ts-p210 Ba/F3 cells, activation of the MAP kinase (ERK 2) signal transduction pathway in ts-p210 Ba/F3 cells is not an immediate and early consequence of BCR−ABL kinase activation, reflecting its apparent failure to deliver a rapid proliferative stimulus in Ba/F3 cells.

Results

Construction of a temperature sensitive p210 BCR−ABL kinase mutant

Various temperature sensitive mutants of the AbMLV protein tyrosine kinase, v-ABL, have been generated by *in vitro* mutagenesis and site-directed mutagenesis (Kipreos *et al.*, 1987; Takemori *et al.*, 1987; Kipreos and Wang, 1988; Cleveland *et al.*, 1989; Oka *et al.*, 1989). One such v-ABL mutant contains a methionine insertion (Kipreos *et al.*, 1987) and another a lysine to glycine substitution (Engelman and Rosenberg, 1987) within the kinase domain. We introduced both mutations into p210 BCR−ABL and expressed the mutant protein (ts-p210) in the IL-3 dependent cell line, Ba/F3. This murine bone marrow derived pro-B lymphoblastoid cell line is strictly dependent upon IL-3 for survival and proliferation, and cells die by apoptosis following IL-3 withdrawal (Palacios and Steinmetz, 1985; Rodrigeuz-Tarduchy *et al.*, 1990). It has previously been shown that expression of p210 BCR−ABL confers growth factor independence to these cells (Daley and Baltimore, 1988).

Following antibiotic selection and cloning by limiting dilution at 38°C, four Ba/F3 clones expressing the temperature sensitive mutant p210 BCR−ABL protein (ts-p210 Ba/F3 #1 to #4) were analysed further. The level of ts-p210 expression was assessed by Western blotting with an anti-ABL antibody (Figure 1). All four clones expressed similar levels of ts-p210. Furthermore, they demonstrated temperature dependent ts-p210 mediated tyrosine phosphorylation of cellular proteins. Each clone was incubated in the absence of IL-3 at 38 or 32°C for 2 h. Total cellular proteins were run on SDS−PAGE and Western blotted with an anti-phosphotyrosine antibody (Figure 2). In ts-p210 Ba/F3 cells incubated at 38°C, the levels of cellular phosphotyrosine were very low, similar to those of parental Ba/F3 cells at either 38 or 32°C in the absence of IL-3. Two hours following a temperature shift to 32°C, however, ts-p210 Ba/F3 cells contained greatly elevated levels of cellular phosphotyrosine, comparable with those of wild-type p210 BCR−ABL (wt-p210) expressing Ba/F3 cells (Figure 2). Western blotting of cellular lysates derived from ts-p210 Ba/F3 cells maintained at 38 or 32°C with an ABL specific antibody reveals that the observed effects are not due to a temperature effect upon the stability of the ts-p210 protein (Figure 1).

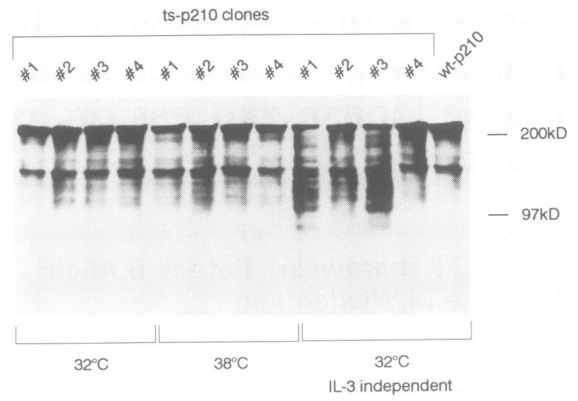

Fig. 1. ts-p210 expression levels in ts-p210 Ba/F3 clones. Anti-ABL Western blot of total cellular lysates from ts-p210 Ba/F3 cells incubated for 2 h at 38 and 32°C, IL-3 independent ts-p210 Ba/F3 cells and wt-p210 Ba/F3 cells maintained at 32°C. The blots were incubated with anti-ABL antibody then a secondary antibody conjugated to horseradish peroxidase and exposed to ECL detection reagents followed by autoradiography for 10 s. The levels of ts-p210 protein are not significantly affected by temperature. The development of IL-3 independence in ts-p210 Ba/F3 cells is not associated with selection of cells expressing higher levels of ts-p210 protein.

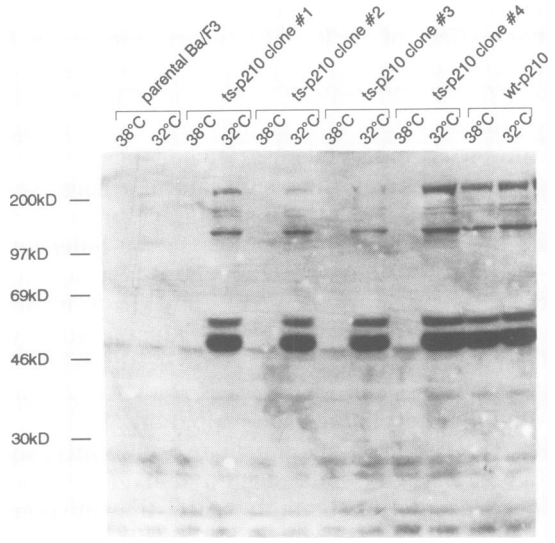

Fig. 2. Analysis of tyrosine phosphorylated proteins in ts-p210 Ba/F3, wt-p210 Ba/F3 and Ba/F3 cells. Anti-phosphotyrosine Western blot of total cellular lysates from Ba/F3, ts-p210 Ba/F3 and wt-p210 Ba/F3 cells maintained at 38°C or 32°C for 2 h in the absence of IL-3. Lysates, normalized for protein content, were run on SDS−PAGE and transferred onto PVDF membrane. The blot was incubated with anti-phosphotyrosine antibody then a secondary antibody conjugated to horseradish peroxidase and exposed to ECL detection reagents followed by autoradiography for 30 s. At 38°C, ts-p210 Ba/F3 cells contain levels of phosphotyrosine comparable to those of Ba/F3 cells, demonstrating that ts-p210 BCR−ABL tyrosine kinase activity is completely suppressed at the non-permissive temperature.

We performed a temperature-shift experiment with ts-p210 Ba/F3 #1 cells, sampling cells at various times following temperature switch, to determine the rate of ts-p210 kinase activation and suppression. Western blotting with an anti-phosphotyrosine antibody demonstrates that

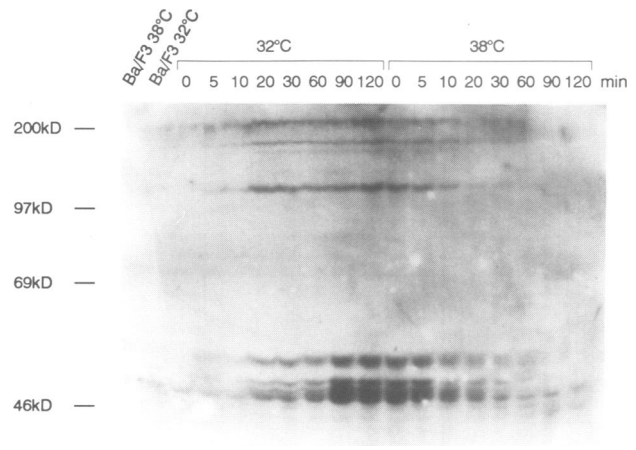

Fig. 3. Time-course of ts-p210 BCR−ABL tyrosine kinase activation and suppression. Anti-phosphotyrosine Western blot of total cellular lysates from ts-p210 Ba/F3 #1 cells maintained at 32 or 38°C in the absence of IL-3 for the indicated times. For each time-point, 0.5×10^6 cells were incubated at 38°C or 32°C for 2 h and then shifted to the other temperature for the times indicated prior to sampling. Tyrosine phosphorylation of cellular proteins by ts-p210 BCR−ABL is rapidly modulated by temperature shift.

ts-p210 kinase activity is rapidly modulated upon temperature shift (Figure 3). Increased tyrosine phosphorylation of cellular proteins is evident within 10 min of a temperature shift to 32°C whilst 10 min following a shift to 38°C, protein tyrosine phosphorylation is already markedly reduced.

Growth factor requirement of ts-p210 Ba/F3 cells

ts-p210 Ba/F3 clones #1 to #4 were also assayed for their ability to survive and proliferate in the absence of IL-3. Unlike wt-p210-expressing Ba/F3 cells, none of the ts-p210 Ba/F3 clones displayed IL-3 independence, although all showed prolonged survival in the absence of IL-3 at 32°C compared with parental or mock-transfected Ba/F3 cells (Figure 4). Whilst Ba/F3 cells underwent apoptotic cell death with complete loss of viability 48 h following IL-3 withdrawal at 32°C, ts-p210 Ba/F3 cells were partially protected from apoptosis. A small proportion of cells remain viable 4–5 days following IL-3 withdrawal at 32°C (2–4% of the starting population). Analysis of genomic DNA from cells following their incubation in the absence of IL-3 for 18 h at 32°C demonstrates that, unlike the ts-p210 Ba/F3 cells, the parental Ba/F3 cells have suffered internucleosomal DNA fragmentation characteristic of apoptotic cells (Figure 5a). The immediate consequence of p210 BCR−ABL expression in Ba/F3 cells is to delay apoptosis which occurs following IL-3 withdrawal. At 38°C, ts-p210 Ba/F3 cells die by apoptosis in the absence of IL-3 similarly to parental Ba/F3 cells (Figures 4 and 5b).

The expression of wt-p210 in Ba/F3 cells renders them completely IL-3 independent at either 32 or 38°C (Figure 4f). These cells may have acquired growth factor independence during antibiotic selection and cloning following transfection, during which time (3–4 weeks) cells are continuously exposed to BCR−ABL tyrosine kinase activity. We therefore sought to determine whether ts-p210 Ba/F3 cells evolve to IL-3 independence during prolonged culture at 32°C in the presence of IL-3, conditions which do not positively select for IL-3 independence.

Cells from ts-p210 Ba/F3 cultures maintained at 32°C in the presence of IL-3 were sampled at various intervals and examined for their ability to survive and proliferate in the absence of IL-3. We found that cultures became dominated by cells capable of IL-3 independent survival and very slow growth under these conditions. These cultures eventually comprised cells which were completely IL-3 independent for both survival and proliferation. All four ts-p210 Ba/F3 clones eventually acquired complete IL-3 independence, although they varied in the length of time required (Figure 4). Clones #3 and #4 were capable of proliferating at similar rates in the presence or absence of IL-3 after only 14 days, while clones #1 and #2 displayed an intermediate behaviour at 14 days, and only after 30 days displayed a fully growth factor independent phenotype. This time related behaviour was reproducible for each of the four clones. As the four ts-p210 Ba/F3 clones express similar levels of ts-p210 protein (Figure 1), the time required for growth factor independence to emerge is not related to the level of BCR−ABL expression.

The small proportion (2–4%) of ts-p210 Ba/F3 cells remaining viable following IL-3 withdrawal at 32°C failed to expand significantly in culture. These cells were expanded for five days at 38°C in IL-3 containing medium and subsequently cultured in the absence of IL-3 at 32°C. Cells retained their capacity to survive IL-3 deprivation, and proliferated very slowly with a doubling time of ~7 days (Figure 6a). FACS analysis of these cells (Figure 6b) following propidium iodide staining showed no evidence of apoptosis or cell-cycle arrest, although compared with parental Ba/F3 cells growing in the presence of IL-3 (Figure 6c), there is a moderate accumulation of cells within the G_2/M compartment, possibly reflecting an elongated G_2/M phase. Nevertheless, the continued culture of these surviving cells at 32°C in the absence of IL-3 eventually gave rise to cells capable of IL-3 independent proliferation. These were shown to be completely growth factor independent by virtue of their ability to proliferate at comparable rates in the presence or absence of IL-3 at 32°C.

There is again variability amongst the four ts-p210 Ba/F3 clones with respect to the time taken for IL-3 independent cells to emerge; clones #1 and #2 gave rise to proliferating cells ~25 days, and clones #3 and #4 ~10 days, following IL-3 withdrawal and temperature shift to 32°C. When cells were seeded into wells of a 24-well plate (0.5×10^6 cells in 1 ml) in the absence of IL-3 and maintained at 32°C, within each ts-p210 Ba/F3 clone, all wells became saturated with proliferating cells following similar times in culture to those described above (data not shown), demonstrating the reproducibility of our observations. Thus, complete IL-3 independence arose following times in culture similar to those required for its development during culture in the presence of IL-3.

These results demonstrate that relief of growth factor dependence is not a primary effect of BCR−ABL expression, at least in Ba/F3 cells; cells acquire a capacity for IL-3 independent proliferation during prolonged exposure to BCR−ABL kinase activity. The primary consequence of BCR−ABL expression in Ba/F3 cells is to moderate suppression of apoptosis following IL-3 withdrawal.

We have maintained cultures of growth factor independent ts-p210 Ba/F3 cells at 32°C in the absence of

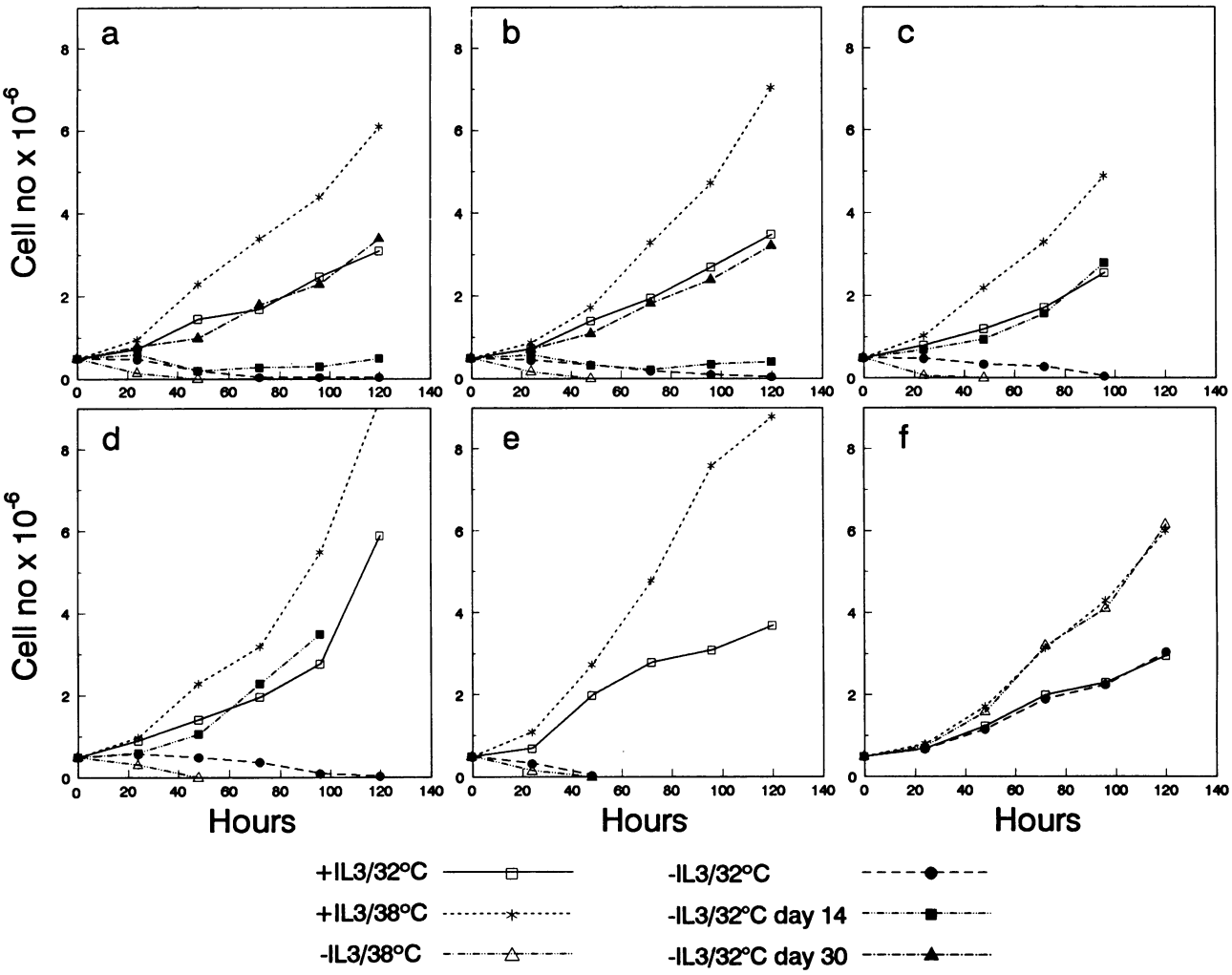

Fig. 4. Growth curves for Ba/F3, wt-p210 Ba/F3 and ts-p210 Ba/F3 cells. Cells were seeded at 0.5×10^6 per ml in RPMI/10% FCS and fed with FCS, and IL-3 where appropriate, every 3 days. Compared with Ba/F3 cells, cell death associated with IL-3 deprivation is suppressed in ts-p210 Ba/F3 cells. In addition, ts-p210 Ba/F3 cultures were maintained in the presence of IL-3 at the permissive temperature for 14 (day 14) or 30 (day 30) days prior to evaluation of their growth potential in the absence of IL-3. Viable cells were counted every 24 h. Cultures become dominated by cells capable of IL-3 independent growth during exposure to ts-p210 tyrosine kinase activity. The parental Ba/F3 cells retain their requirement for IL-3 following prolonged culture at 32°C (data not shown). (**a**) ts-p210 Ba/F3 #1; (**b**) ts-p210 Ba/F3 #2; (**c**) ts-p210 Ba/F3 #3; (**d**) ts-p210 Ba/F3 #4; (**e**) Ba/F3 and (**f**) wt-p210 Ba/F3. Data shown are representative of three independent experiments. In (a–d) the clones response to shift to the non-permissive temperature of 38°C in the absence of growth factor does not change following prolonged culture at the permissive temperature and development of growth factor independence; thus, the data for this line are the average and are representative of the clones behaviour.

IL-3 for up to three months, and these cells retain an absolute requirement for BCR–ABL kinase activity for IL-3 independent proliferation; temperature shift to 38°C results in cell death by apoptosis, demonstrating that BCR–ABL initiates, and contributes to the maintenance of, the growth factor independent state in Ba/F3 cells (Figure 4 and data not shown). We have found that conditioned medium from IL-3 independent ts-p210 Ba/F3 cells fails to support the survival and growth of parental Ba/F3 cells (data not shown) suggesting that growth factor independence does not arise via an autocrine mechanism.

ts-p210 BCR–ABL does not activate the MAP kinase pathway in Ba/F3 cells

IL-3 stimulation of an IL-3 dependent cell line has been shown to result in the activation of the extracellular signal regulated kinase, ERK 2 (Okuda *et al.*, 1992). ERK 2 is activated in response to IL-3 stimulation of Ba/F3 cells and is constitutively active in Ba/F3 cells maintained

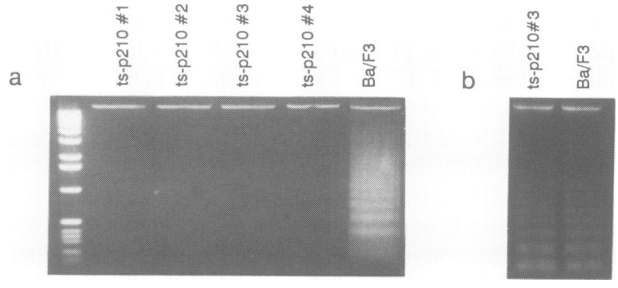

Fig. 5. Analysis of genomic DNA by agarose gel electrophoresis from cells cultured for 18 h at 32°C in the absence of IL-3 (**a**) and for 14 h at 38°C in the absence of IL-3 (**b**). Apoptosis induced by IL-3 withdrawal is delayed in cells expressing ts-p210.

in IL-3 (Figure 7 and our unpublished observations). BCR–ABL kinase does not appear to deliver a proliferative signal in Ba/F3 cells. We sought to confirm this by

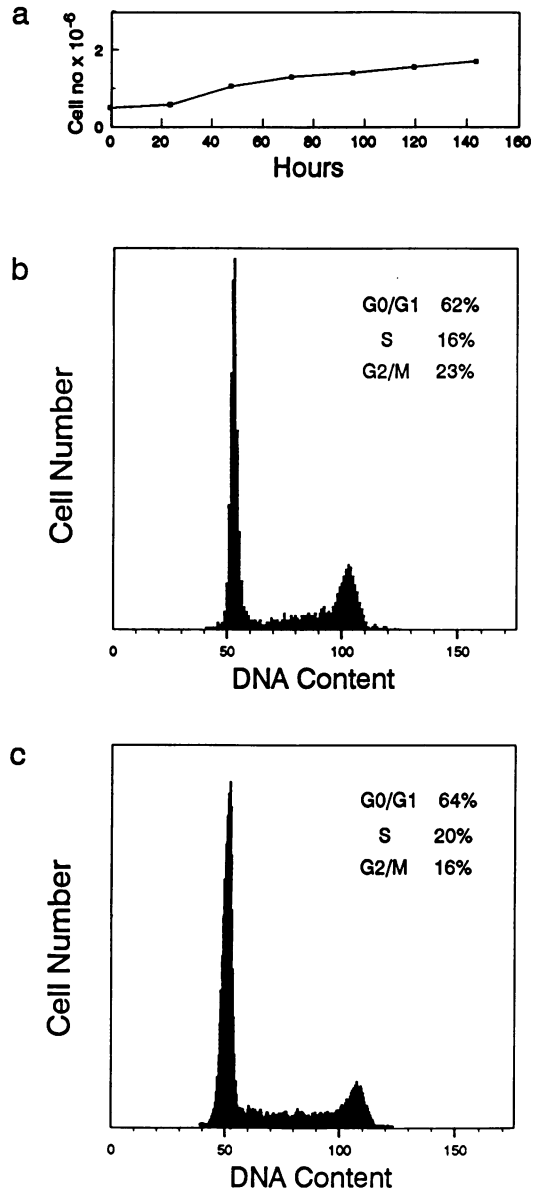

Fig. 6. Analyses of rescued ts-p210 Ba/F3 cells. The minor population of ts-p210 Ba/F3 #1 cells remaining viable 10 days following IL-3 withdrawal at 32°C were expanded in IL-3 containing medium at 38°C for 5 days. Cells were subsequently cultured at 32°C in the absence of IL-3; viable cell counts were performed every 24 h (**a**). In addition, cells were sampled at 3 days for cell-cycle analysis by FACS (**b**), as were parental Ba/F3 cells growing in the presence of IL-3 at 32°C for 3 days (**c**). Cells proliferate very slowly and there is no evidence of cell-cycle arrest or apoptosis, demonstrating that all cells within the initial population are capable of IL-3 independent survival. The proportion of cells within each cell-cycle compartment is indicated.

analysing the status of ERK 2 in response to activation of BCR–ABL kinase by temperature shift of ts-p210 Ba/F3 cells. ts-p210 Ba/F3 cells were deprived of IL-3 and maintained at 32 or 38°C for 12 h. Cells were lysed, ERK 2 protein was immunoprecipitated and subjected to an *in vitro* kinase reaction with myelin basic protein (MBP) as a substrate. Reactions were run on SDS–PAGE and the dried gel visualized by autoradiography. ^{32}P incorporation into MBP was measured using a phosphorimaging system. In addition, lysates were Western blotted with an antibody to ERK 2; activation of ERK 2 is associated with the presence of a slower migrating phosphorylated form of the protein (Figure 7). We observe no ERK 2 activation above that in parental Ba/F3 cells in response to ts-p210 kinase expression, showing that in Ba/F3 cells, p210 BCR–ABL does not signal activation of the MAP kinase pathway. In addition, we observe no activation of ERK 2 in ts-p210 Ba/F3 at earlier time-points (up to 2 h) following their shift to the permissive temperature (data not shown).

ERK 2 is constitutively activated in IL-3 independent cells derived from ts-p210 Ba/F3 clones, consistent with their capacity for IL-3 independent proliferation (Figure 8). In parental Ba/F3 cells, IL-3 deprivation for 1 h results in dramatic reduction of ERK 2 activity (data not shown). However, shift of IL-3 independent ts-p210 Ba/F3 cells, proliferating in the absence of IL-3 at 32°C, to the non-permissive temperature for 4 h, does not result in a concomitant reduction of ERK 2 kinase activity, although these cells are destined to die by apoptosis. This shows that development of IL-3 independence in ts-p210 Ba/F3 cells is not directly associated with coupling of BCR–ABL to the MAP kinase pathway during their adaptation to ts-p210 BCR–ABL tyrosine kinase activity. Thus we propose that activation of the MAP kinase pathway in p210 BCR–ABL expressing Ba/F3 cells is solely a consequence of their growth factor independence and is not a primary consequence of p210 BCR–ABL expression.

BCR–ABL does not activate the MAP kinase pathway in COS cells

To show unequivocally that BCR–ABL does not signal activation of the MAP kinase pathway, and so rule out the possibility that the ts-p210 protein is compromised with respect to its interaction with critical signalling molecules, we co-expressed wild-type p210 BCR–ABL and myc-epitope tagged ERK 2 (mycERK 2) in COS-1 cells and analysed the kinase activity of the immunoprecipitated mycERK 2 protein towards MBP. We find that expression of p210 BCR–ABL results in negligible activation of mycERK 2, confirming that IL-3 and BCR–ABL mediated signals differ with respect to their ability to activate the MAP kinase pathway (Figure 9). We also demonstrate a fundamental difference between BCR–ABL and v-ABL mediated signalling in COS cells; v-ABL expression results in a ~4-fold activation of mycERK 2 (Figure 9).

Discussion

Temperature sensitive mutants of tyrosine kinase oncogenes have proved invaluable in determining the primary biological effects of their expression and, importantly, to distinguish these from consequences of cellular transformation. For example, expression of ts v-ABL in NIH 3T3 cells demonstrates that v-ABL has a cytostatic effect on most cells, with only a minority amenable to transformation (Renshaw *et al.*, 1992). Toxic or cytostatic effects of oncogenic forms of ABL have been indirectly observed (Jackson and Baltimore, 1989), but a system in which expression of ABL tyrosine kinase activity is inducible allows one to examine such deleterious effects, which otherwise remain ill-defined; clonal v-ABL expressing cell lines can be established without prior exposure to its

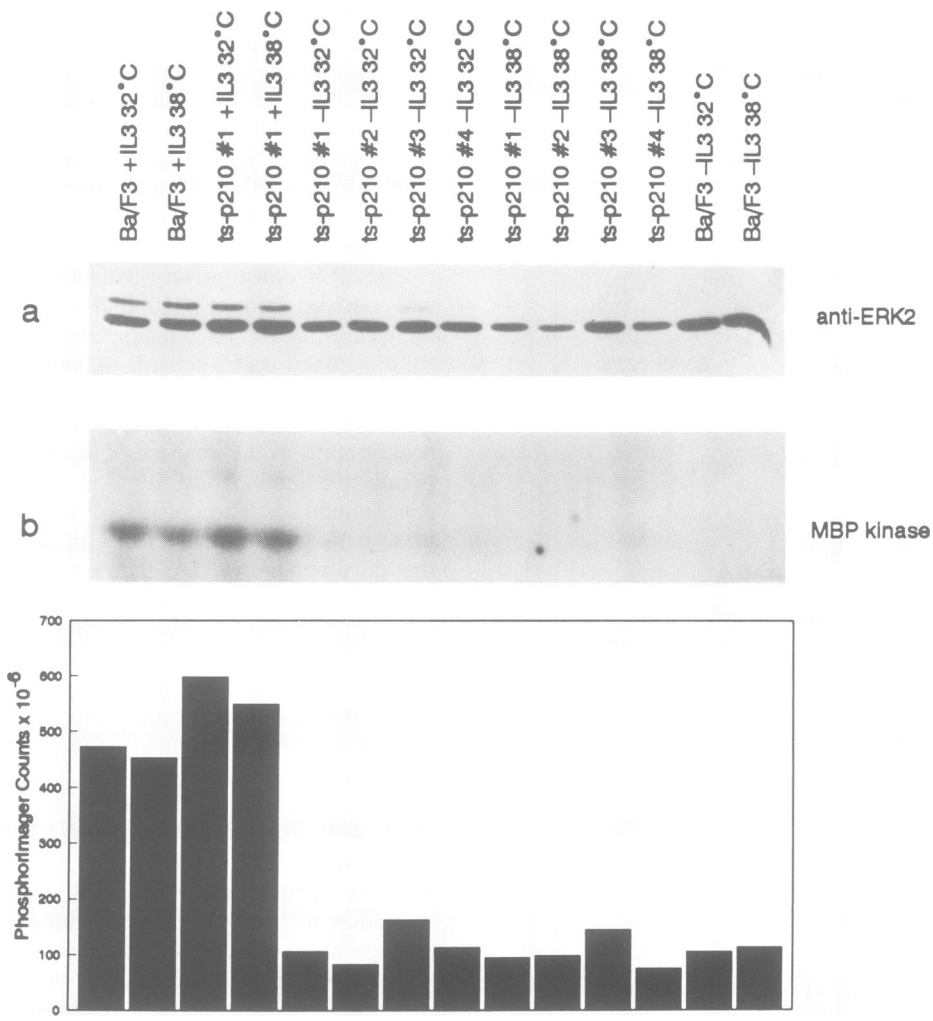

Fig. 7. ERK 2 activity in ts-p210 BCR−ABL expressing Ba/F3 cells. (**a**) Anti-ERK 2 Western blot of total cellular lysates from Ba/F3 or ts-p210 Ba/F3 cells following incubation at 32°C or 38°C in the presence or absence of IL-3 for 12 h (as indicated). (**b**) MBP kinase assays were performed on ERK 2 immunoprecipitated from these same lysates. ^{32}P incorporation into MBP was measured using a phosphorimaging system.

tyrosine kinase activity. ts v-ABL mutants have also allowed differences between IL-3 and v-ABL regulated growth to be revealed in factor dependent haematopoietic cell lines (Cleveland *et al.*, 1989; Spooncer *et al.*, 1994).

However, fundamental differences exist between BCR−ABL and v-ABL with respect to their transforming potential in fibroblasts (Daley *et al.*, 1987) and their interaction with signalling proteins (Varticovski *et al.*, 1991). It may not be appropriate, therefore, to draw conclusions regarding mechanisms of BCR−ABL mediated transformation and leukaemogenesis based on experimental results obtained with v-ABL. To obviate this problem we constructed a temperature sensitive BCR−ABL mutant (ts-p210). We used the ts-p210 mutant to determine the immediate response of IL-3 dependent Ba/F3 cells to BCR−ABL expression; we find that the primary effect is a moderate suppression of apoptosis following growth factor withdrawal. A small proportion of cells survive growth factor deprivation and remain viable, eventually acquiring the capacity for IL-3 independent proliferation.

Previous studies have shown that the CML-derived K562 cell line is resistant to apoptosis induced by chemotherapeutic agents and serum deprivation (McGahon *et al.*, 1994); down-regulation of BCR−ABL protein levels in K562 cells through expression of antisense oligodeoxynucleotides markedly increased their susceptibility to apoptosis. The K562 cell line was derived from a CML patient in blast crisis and harbours additional genetic abnormalities, a feature characteristic of the progression of chronic phase CML to blast crisis, thus making it difficult to unequivocally ascribe properties of this cell line solely to BCR−ABL expression. Our results, however, demonstrate that BCR−ABL can function as an anti-apoptotic factor in growth factor dependent haematopoietic cells, and this property may be instrumental in the deregulation of myelopoiesis characteristic of chronic phase CML.

ts-p210 Ba/F3 cultures maintained at the permissive temperature in the presence of IL-3 become dominated by cells capable of survival and very slow proliferation in the absence of IL-3, and these progress to comprise fully growth factor independent cells. It appears, therefore, that Ba/F3 cells require prolonged exposure to BCR−ABL kinase activity before they acquire growth factor independence. Furthermore, active BCR−ABL kinase is required

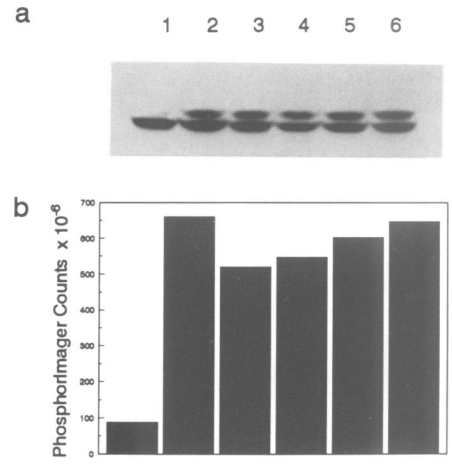

Fig. 8. ERK 2 is constitutively active in growth factor independent ts-p210 Ba/F3 cells. ERK 2 activity in IL-3 independent cells derived from ts-p210 Ba/F3 clones #1 and #3 maintained for 24 h at 32°C in the absence of IL-3 (lanes 3 and 5), and following their subsequent incubation at 38°C for 4 h (lanes 4 and 6). Ba/F3 IL-3 32°C 12 h (lane 1); Ba/F3 +IL-3 32°C 24 h (lane 2). (**a**) anti-ERK 2 Western blot, (**b**) MBP kinase assays.

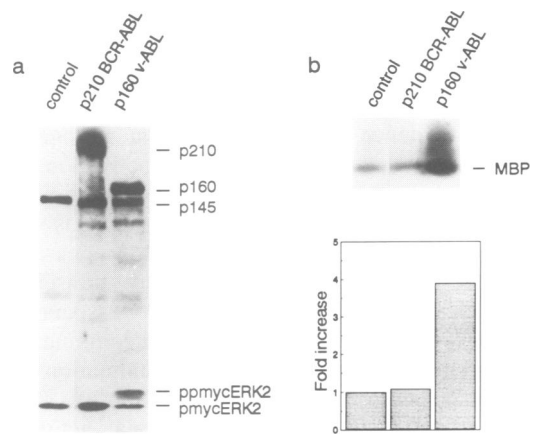

Fig. 9. MBP kinase assays on mycERK 2 immunoprecipitated from COS-1 cells co-expressing ABL oncogenes. (**a**) Western blot of total cellular lysates probed with anti-ABL and anti-myc epitope (9E10) antibodies; (**b**) mycERK 2 MBP kinase assays and ^{32}P incorporation into MBP. Lane 1, mycERK 2 + pBABE puro vector control; lane 2, mycERK 2 + p210 BCR−ABL; lane 3, mycERK 2 + p160 v-ABL. p210 BCR−ABL expression results in no significant activation of mycERK 2 above control.

for the continued survival and proliferation of these cells. Cells shifted to the non-permissive temperature die within 48 h by apoptosis.

ts-p210 Ba/F3 cells evolve to growth factor independence under conditions that do not positively select for its development. In addition, ts-p210 Ba/F3 cells undergo several rounds of cell division during their progression to growth factor independence. It is unlikely, therefore, that a process of adaptation to BCR−ABL mediated signals occurs, in which up- or down-regulation of signalling molecule/s is required co-operatively for abrogation of growth factor dependence. The development of growth factor independence in haematopoietic cells expressing BCR−ABL, v-ABL, or v-Myc and v-Ha-Ras has been reported to be associated with karyotypic instability (Vogt *et al.*, 1986, 1987; Majone *et al.*, 1988; Laneuville *et al.*, 1992). A more likely possibility therefore, is that BCR−ABL expression renders cells genetically unstable, promoting the accumulation of mutations which, in co-operation with BCR−ABL, lead to growth factor independence. Such a mechanism is particularly attractive considering the apparent requirement for additional genetic events in the progression of CML from chronic phase to blast crisis. Karyotypic examination of bone marrow from BCR−ABL transgenic mice has shown that disease progression is associated with the accumulation of chromosomal abnormalities (Voncken *et al.*, 1992). Although associated with cellular transformation, genetic instability may be an initiating event in the transformation of haematopoietic cells by BCR−ABL.

ts-p210 Ba/F3 clones #1 and #2 require a longer period of exposure to ts-p210 tyrosine kinase activity for the development of growth factor independence than clones #3 and #4; this does not appear to be a consequence of lower ts-p210 expression levels (Figure 1). We suggest that this difference in latency may reflect heterogeneity within the Ba/F3 cell line with respect to genetic mutations; a proportion of cells may already contain mutations required in co-operation with BCR−ABL for the abrogation of growth factor dependence.

Recently, Carlesso *et al.* (1994) reported that a temperature sensitive p210 BCR−ABL protein renders murine myeloid IL-3 dependent 32Dc13 cells growth factor independent. Importantly, and in stark contrast to our observations with our temperature sensitive p210 BCR−ABL mutant in Ba/F3 cells, 32Dc13 cells were immediately rendered IL-3 independent upon shift to the permissive temperature; 32Dc13 cells expressing temperature sensitive BCR−ABL behaved identically to wild-type BCR−ABL expressing 32Dc13 cells in the absence of IL-3 at 33°C (the permissive temperature) with respect to their proliferative properties. This may reflect a fundamental difference between 32Dc13 and Ba/F3 cells in terms of their genetic backgrounds; if additional genetic mutations in addition to BCR−ABL expression are required for abrogation of growth factor dependence, 32Dc13 cells may already harbour co-operating mutations. It is apparent, however, that their BCR−ABL mutant protein retains significant residual kinase activity at the restrictive temperature of 39°C, which may be sufficient to promote development of growth factor independence during selection, cloning and maintenance in culture, despite being insufficient to sustain IL-3 independent proliferation.

If BCR−ABL kinase is delivering a proliferative signal, functionally replacing that delivered by IL-3 receptor stimulation, activation of the Ras-dependent MAP kinase pathway should be observed following activation of BCR−ABL tyrosine kinase activity in ts-p210 Ba/F3 cells. We found no such activation and confirmed our observations in COS-1 cells, where BCR−ABL mediates negligible activation of a co-expressed ERK 2 protein.

Receptor tyrosine kinase signals are coupled to the RAS/MAP kinase pathway via the GRB-2 adaptor/mSOS1 guanine nucleotide releasing factor complex (McCormick, 1993). Recently, GRB-2 has been shown to bind Tyr177 within the first exon of BCR−ABL and mutation of this residue abrogates the ability of BCR−ABL to activate

transcription from a promoter containing Ras-responsive elements (ets/AP-1) (Pendergast *et al.*, 1993). In addition, this mutant BCR−ABL protein is severely impaired with respect to lymphoid and fibroblast transformation. However, evidence demonstrating that abrogation of BCR−ABL mediated transcriptional activation by co-expression of the dominant negative N17 Ras mutant was due entirely to impairment of endogenous Ras function was not presented. It is possible that the apparent Ras-dependence of BCR−ABL mediated transcriptional activation simply reflects cellular toxicity associated with N17 Ras expression (S.Leevers, personal communication). In the absence of evidence for the accumulation of Ras-GTP in response to BCR−ABL expression it is therefore possible that the observed transcriptional activation is mediated by other signalling protein/s. The abrogation of transcriptional activation by mutation of the Tyr177 GRB-2 binding site in BCR−ABL could be the result of the inability of signalling proteins other than GRB-2 to bind BCR−ABL, either at Tyr177, or at one of several other phosphotyrosine residues within the first exon of BCR whose availability for binding to critical target proteins may be perturbed as a result of the mutation; this would presumably also impair BCR−ABL mediated transformation. In addition, other as yet unidentified GRB-2 binding proteins may be involved in the BCR−ABL mediated transformation pathway.

It is assumed that localization of mSOS-1 to the plasma membrane is sufficient to mediate activation of the Ras pathway. The absence of membrane directing sequences in BCR−ABL raises the question of how it could mediate Ras activation by recruitment of GRB-2/mSOS 1 to the plasma membrane; BCR−ABL is localized in the cytoplasm, where it binds strongly to actin (McWhirter and Wang, 1991, 1993). Interestingly, we find that v-ABL mediates activation of a coexpressed ERK 2 protein in COS cells. The myristoylation signal present at the amino terminus of the GAG moiety in v-ABL may mediate plasma membrane localization appropriate for Ras-dependent activation of the MAP kinase pathway.

Our results suggest that the reported elevated ratio of Ras bound GTP/GDP in growth factor independent myeloid cells (Mandanas *et al.*, 1993) may be a consequence of BCR−ABL mediated transformation but not necessarily a primary consequence of BCR−ABL expression. Indeed, we have shown that activation of the MAP kinase pathway in growth factor independent BCR−ABL expressing Ba/F3 cells is only a consequence of their IL-3 independent proliferation. However, it is possible that a low level of BCR−ABL is present at the plasma membrane, for example at adhesion plaques, and signals a low level of Ras activation, insufficient for MAP kinase activation, but nevertheless functionally important with respect to activation of other downstream signalling pathways leading to transformation.

It appears, therefore, that BCR−ABL may activate only a subset of IL-3 mediated signals in Ba/F3 cells, with those constituting a survival signal probably of primary importance in the suppression of apoptosis associated with BCR−ABL expression. We conclude that a role for BCR−ABL kinase in the constitutive activation of tyrosine kinase-dependent components of the IL-3 mediated signal may not be of primary importance, and the relief of growth factor dependence in IL-3 dependent haematopoietic cell lines does not simply reflect convergence of BCR−ABL and IL-3 signalling pathways.

Our results suggest that the myeloid expansion characteristic of chronic phase CML may result from prolongation of cell survival rather than deregulated proliferation. We suggest that BCR−ABL expression alters the normal developmental controls in leukaemic progenitor cells by suppressing apoptosis, effectively increasing the pool of cells susceptible to other mutagenic events which ultimately lead to progression to blast crisis. Expression of BCR−ABL may accelerate the mutagenic events in these cells through induction of genetic instability.

Materials and methods

Plasmids

The p210 BCR−ABL cDNA was provided by Gerard Grosveld and the p160 v-ABL cDNA by Jean Wang. Mutations were introduced into the p210 BCR−ABL coding sequence using the M13 based Mutagenesis Kit (Amersham, RPN.1523). All manipulations were performed on M13 containing an 880 bp Asp718−*Aat*II fragment from the ABL kinase domain coding sequence. An ATG codon was inserted following nucleotide 1128 in the c-ABL cDNA resulting in a methionine insertion. Nucleotides 1391 and 1390 were substituted (A to G) resulting in a lysine to glycine substitution (numbering as in sequence HSABL in GenBank).

cDNAs were adapted at their 3′-termini with an *Mlu*I linker and inserted into the MPSV based retroviral expression vector pM5Neo (Laker *et al.*, 1987) (modified by insertion of a *Mlu*I linker into the *Bgl*II site) for stable transfection into Ba/F3 cells, and into the SV40 ori containing pBABE puro vector (Morgenstern and Land, 1990) for transient expression in COS-1 cells (Landau and Littman, 1992).

Cell culture

Ba/F3 (Palacios and Steinmetz, 1985) and ts-p210 Ba/F3 cell lines were maintained in a 5% CO_2-gassed incubator at 38°C unless otherwise indicated. COS-1 cells were maintained in DMEM and 10% FCS in a 5% CO_2-gassed incubator at 37°C.

Ba/F3 cells were maintained in RPMI supplemented with 10% fetal calf serum (FCS) and 2% mIL-3 conditioned medium. The IL-3 producing cell line was kindly provided by Professor Fritz Melchers (Karasuyama and Melchers, 1988).

Electroporation of Ba/F3 cells

Ba/F3 cells (0.5×10^6) were resuspended in 800 μl RPMI and electroporated (250 V, 500 μF) with 10 μg plasmid DNA using a Bio-Rad Gene Pulser®. Following electroporation, cells were seeded into 96-well plates in IL-3-containing medium and maintained at 38°C; 48 h later G418 was added to 1 mg/ml. G418 resistant clones were expanded for subsequent analyses.

COS cell transfection and MBP kinase assay

COS-1 cells were cotransfected with 5 μg pEXV3 ERK2tag and 5 μg pBABE puro encoding BCR−ABL or v-ABL using a standard calcium phosphate technique. Twenty four hours following transfection, cells were deprived of serum and incubated for a further 24 h. The cells were subsequently washed twice with ice-cold phosphate-buffered saline A (PBSA), scraped off the plate in PBSA and collected by centrifugation. Cell pellets were resuspended in 20 mM Tris pH 8.0, 40 mM $Na_4P_2O_7$, 50 mM NaF, 5 mM $MgCl_2$, 0.1 mM Na_3VO_4 and 10 mM EGTA. The cell suspension was made to 1% Triton X-100, 0.5% sodium deoxycholate, 20 μg/ml trypsin inhibitor, 20 μg/ml leupeptin, 20 μg/ml aprotinin and 3 mM PMSF, incubated on ice for 15 min, centrifuged at 10 000 *g* for 5 min and supernatants, normalized for protein content, were subjected to immunoprecipitation with the myc-epitope specific mouse monoclonal antibody 9E10 (Evan *et al.*, 1985). Immunoprecipitates, captured on protein−G Sepharose, were washed twice in lysis buffer without $MgCl_2$ and once in 30 mM Tris pH 8.0. The beads were incubated in a 30 μl reaction volume containing 30 mM Tris pH 8.0, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μM ATP, 0.4 μCi [γ-^{32}P] ATP and 7.5 μg myelin basic protein (MBP) for 30 min at 30°C. Endogenous ERK 2 activity was assayed in Ba/F3 cells by immunoprecipitation

with rabbit antiserum 122 against ERK 2 (a gift from C.J.Marshall). Immunoprecipitates were captured on protein–A Sepharose and subjected to MBP kinase reactions as described above. Reactions were centrifuged and supernatants (25 µl) were transferred into 100 µl of reducing sample buffer. Samples were run on 10% SDS–PAGE and dried gels visualized by autoradiography. In addition, ^{32}P incorporation into MBP was measured using a PhosphorImager (Molecular Dynamics).

Western blotting

For Western blotting with anti-ABL and anti-phosphotyrosine antibodies, cells were resuspended in lysis buffer containing 10 mM phosphate pH 7, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.05% SDS, 20 mM NaF, 1 mM $NaVO_3$, 0.005 mM molybdic acid, 60 µg/ml soybean trypsin inhibitor, 60 µg/ml leupeptin, 60 µg/ml bestatin and 1 mM PMSF. Lysates were normalized for protein content using the Bio-Rad protein assay kit and equal amounts of protein were separated on 7% SDS–PAGE. Proteins were transferred onto PVDF membrane (Immobilon™-P from Millipore) and probed with a mouse monoclonal antibody against ABL (Ab-3 from Oncogene Science) or a mouse monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology, Inc.).

For analysis of ERK 2 and mycERK 2 by western blotting (Leevers and Marshall, 1992), portions of protein lysate to be immunoprecipitated for MBP kinase assay were run on 15% SDS–PAGE, transferred onto PVDF membrane and probed with rabbit antiserum 122 (anti-ERK 2) or 9E10 (anti-myc) monoclonal antibody respectively.

FACS analysis

Cells were fixed in 500 µl of 70% ethanol and resuspended in 1 ml PBSA containing 20 µg propidium iodide and 100 µg RNase. This suspension was incubated at 37°C for 30 min and subsequently analysed using a FACScan® (Becton Dickinson & Co., Mountain View, CA); apoptotic cells are detected in the red fluorescent sub-G1 peak.

DNA fragmentation analysis

Cells were lysed in 10 mM Tris, 1 mM EDTA containing 100 µg/ml proteinase-K and 0.5% SDS. Following overnight incubation at 37°C, DNA was prepared by phenol:chloroform extraction. DNA was resolved by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining.

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